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13. ABSTRACT *(Maximum 200 Words)*

Estrogen receptor alpha (ER) plays an important role in the development and progression of breast cancer, and it is routinely used as a marker for hormone sensitivity in breast cancer patients (1). Positive ER status is a useful indicator for a first-line therapy with antiestrogens (2).

ER is expressed from at least two promoters (Fig.1). The resulting transcripts from these two promoters differ only in the non-coding region upstream of the major ER open reading frame (ORF); the ER proteins from these two promoters are identical.

The proximal promoter transcript contains a 20 residue ORF which closes 52 nt upstream of the main ER ORF. which affects expression from the downstream ER ORF. **Our central hypothesis is that the action of the proximal transcript uORF is exerted at the *translation* level.** C-terminal truncated (Phe20 or Gly 19 to stop) prox-uORFs are highly effective and enhanced translational inhibitors. The presence of the natural C-terminal residue of the proximal uORF modifies (weakens) the inhibitory potential of the uORF. An ongoing goal aim is to under define critical regions for the inhibitory effect and to address mechanism..

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Antibody Probes to transcript-specific peptides provide a novel tool to investigate the role of alternate Estrogen Receptor promoter use in breast cancer
DAMD 17 01 1 0529 Principal Investigator: Pentecost, Brian T.

INTRODUCTION

Estrogen receptor alpha (ER) plays an important role in the development and progression of breast cancer, and it is routinely used as a marker for hormone sensitivity in breast cancer patients (1). Positive ER status is a useful indicator for a first-line therapy with anti-estrogens (mainly Tamoxifen) (2).

ER is expressed from at least two promoters (Fig.1). The resulting transcripts from these two promoters differ only in the non-coding region upstream of the major ER open reading frame (ORF); the ER proteins from these two promoters are identical. The distal promoter is 2 Kbp upstream of the main translational start of the ER ORF and is spliced into nt164 of the proximal form. Both proximal and distal ER promoter transcripts have short peptide ORFs in the unique regions upstream of ER itself (Fig 1.b). The proximal promoter transcript contains a 20 residue ORF (ER Prox-uORF) which closes 52 nt upstream of the main ER ORF. The distal promoter transcript also contains an 18 residue uORF that shares 5 C-terminal codons with the prox-peptide of the proximal promoter transcript as splicing to shared sequence occurs within the uORF region.

Our preliminary data indicated that the proximal transcript uORF affects expression from the downstream ER ORF, while the equivalent distal transcript ORF did not in a flow cytometry assay. **Our central hypothesis is that the action of the proximal transcript uORF is exerted at the *translational* level.**

A loose consensus for the region around the eukaryotic initiator AUG was defined almost twenty years ago (3). Strong start sites (Kozak consensus) tend (95%) to have purines (normally A) at -3 position relative to the AUG, and G at +4 position (see Fig1.b). The basic scanning model of eukaryotic translation machinery presumes that the first 5' AUG in a sequence should encode the translational initiator methionine (4). So any upstream ORF should be expressed to some degree and have potential influence on the downstream expression in which the mechanism is poorly understood. It is well known that re-initiation is required before the downstream translational start codon can be utilized. An example of that is the *S cerevisiae* GCN4 gene (5). In addition, translational regulation of specific mRNAs has been studied and is generally exerted through *cis*-acting elements in the mRNAs. One example of that is iron regulatory proteins which inhibit ferritin translation by binding to specific 5' UTR sites (6). The studied ORFs (5 principal eukaryotic examples) lack conserved (shared) features (7) and lack homology to ER ORFs.

BODY

Please note that the goals of this concept project where changed in coordination with BCRP program staff to avoid overlap with a concurrent IDEA to Dr Pentecost. Modified abstracts and statements of work were filed prior to the project opening.

Task 1(from revised statement of work). *Carry out mutagenesis of 20 residue upstream Open Reading Frame (uORF) of the Estrogen Receptor (ER) proximal promoter transcript. Targets will address the role of hydrophobicity and charge in the action of the peptide on translation, in addition any role for the terminal phenylalanine codon will be specifically studied. Where changes affect inhibitory action, then additional changes will be made which are equivalent in effects on peptide sequence but involve alternate codon use at the nucleic acid level. This will determine if changes are due to effects on peptide sequence rather than on RNA conformation: Studies will continue to use GFP fusions as a reporter unless results of task 3 indicate that a better signal can be obtained with intact ER.*

A series of 6 mutations was made in the unique region of the ER proximal promoter transcript uORF (Prox-uORF), *i.e.* in those regions not shared with the ORF in the distal promoter transcript upstream sequence. This region was targeted because we knew that the uORF of the distal ER trancript was not inhibitory by statistical

tests in a flow cytometry based GFP assay (though a western blot assay suggested some inhibition). It was therefore reasonable to infer that unique regions of the Prox-uORF were the source of its action. These changes were made in the basic ER construct that we have used to study ER translation control, *i.e.* a fusion of the prox ER sequences to GFP. The ER transcript sequences begin upstream of the translational start region for the uORF and conclude with the first 18 codons of the main ER fused in frame to GFP. the ORF is in its natural relationship to the main ER start and closes 52 bp upstream of the ER start. These changes included single mutants (Arg 2 to Gln, Cys 11 to Ser, Arg15 to Ala, Trp 16 to Ala and Pro17 Ala) and a double mutant (Arg 2 to Gln and Arg15 to Ala, see numbering and sequence in Fig. 1B) These changes mainly affect charge and hydrophobicity. The effect of these mutations was equivocal by flow and western blot analysis (data not shown).

The experimental approach was reassessed and we targeted the ORF with a series of C-terminal truncating mutations. We already knew that elimination of the uORF translational start or conversion of Cys3 to a stop (TGA terminator) led to increased downstream ER-GFP expression (these are the original observations leading to the project, also see current data in Figs 3 and 4). Conversion of Cys 11 and Trp 16, separately, to termination codons gave somewhat increased downstream expression (not shown), this is similar to data on eliminating the uORF or truncation at residue Cys 3. In contrast; the uORF was very strongly inhibitory in HeLa cells by the flow cytometry assay when the terminal Phe 20 codon was mutated to a terminator shortening the uORF by one residue (see cartoon in Fig. 2), much more so than the uORF of the parent prox ER-GFP construct (see tracks 2 and 7 in Fig. 3). Substitutions of the Phe codon by Tyr (conservative change) or Ala (more radical) did not strongly affect the inhibitory action of the uORF as compared to the original construct *i.e.* did not enhance inhibition unlike the stop mutant (data not shown). The strong inhibitory effect of the uORF region does seem to be via the uORF (rather than say a conformational effect on RNA structure) since elimination of the uORF by mutational elimination of the of the translational start (ATG to CTG) again led to relief of the inhibition of GFP expression and increased GFP expression to levels above those with unmodified starting ER-prox-GFP construct. the resulting level of GFP expression was similar when translational start of either the truncated uORF or the parent uORF was eliminated (see Fig 3 tracks 1& 3).

Our focus is on the action of the uORF peptide in affecting translation of the ER-prox transcript however, this could clearly involve factors beyond simply the peptide. A concern was that in making the one-codon truncated uORF (Phe 20 deleted) we had introduced an additional stop codon to the 3' region of the uORF and this could be relevant to inhibition. We therefore made an alternate truncated construct where the final (Phe 20) residue was eliminated by 'looping out', removing any codon and leaving a shortened peptide with only the original terminator. The uORF of this sequence was also clearly more strongly inhibitory (see Fig. 5) than that of the parent prox-ER-GFP construct. We are reasonably confident that it is valid to focus on the peptide nature of the uORF in follow up studies.

Further C-Terminal truncation of the uORF by conversion of the (penultimate) uORF Gly 19 codon also clearly increased inhibition of downstream ER-GFP expression (Fig3, track 6 vs 7). The effect was possibly not as great as with the Phe to stop change and better quantitative evaluation will be the focus of further studies.

The potent inhibitory effect of the C-terminal truncated uORF (Phe20 to stop) was eliminated by destroying the translational start of the ORF as noted above (see Fig. 3). As a test of peptide sequence requirements we *reintroduced* translational starts at what would have been residues 10 and 15: This generated uORFs with encoded peptides that were truncated at both the C-and N- terminal ends. These still showed considerable inhibitory effects (see tracks 4 and 5 vs 7 [starting ER-GFP construct] in Fig 3), these data combined with the initial scanning stop study indicate that key inhibitory features may lie to the C-terminal end of the uORF peptide, around residues 16-18.

The Phe to stop mutation was also effective in increasing inhibition by the uORF of the *distal* ER transcript in the flow cytometry assay (not shown): Any hypotheses on *mechanism* will need to address the fact that translational inhibition can occur with an entirely different N-terminal region, albeit with possibly reduced efficiency. It may be that distributed sequences are relevant for establishing the observed levels of

inhibition, in cooperation with critical specific motifs.

In summary, the C-terminal truncated (Phe20 or Gly 19 to stop) prox-uORFs are clearly a highly effective and enhanced translational inhibitors. The presence of the natural C-terminal residue of the proximal uORF modifies (weakens) the inhibitory potential of the uORF. A goal under task 1 will be to better define critical regions and address mechanism.

In our preliminary experiments it was not always clear if variation was due to altered expression of the basic enhanced-GFP reporter or from variable transfection. Several attempt at normalization flow-cytometry samples with co-transfected alternate fluorescent marker were not effective: Enhanced GFP is the only fluorescent reporter that really works in flow cytometry. We therefore focused on normalization in western blot analysis. Our final approach was to generate modified GFP proteins that were of distinct size to that from ER GFP-fusions but which would still interact with GFP antibodies. Several truncated forms of GFP were generated (by C-terminal truncation by introducing a premature stop signal and alternatively by internally looping out an internal C-terminal fragment of the GFP ORF. Both approaches were not satisfactory: Measured expression of the truncated GFPs was poor and we think the modified proteins were unstable. As an alternative we used an N-terminal extended form of GFP, this was the HEM45-GFP construct that was made under prior BCRP support (DAMD17-96-1-6183). This fusion protein has reasonable expression (about 30% of the level of GFP) and a 40K Da mass that is easily resolved from GFP alone or the ER GFP fusion (20 - 22 K Da). It is detected by the same anti-GFP antisera used to detect GFP and ER-GFP fusions.

We have recently begun to repeat analysis of constructs using this co-transfection assay. We tested the approach in three cancer lines using the C-terminal truncated construct set. As seen with the flow cytometry asay in HeLa transfectants; the Phe to stop (residue 20 deleted) uORF mutant clearly caused enhanced inhibition compared to the parent construct in Ishikawa (Uterine, ER+ve adenocarcinoma), MCF-7 (Breast ER+ver adenocarcinoma) and MDA-MB-23 cells (ER-ve breast tumor line) (Fig 4, track 2 vs 4). Elimination of the wild type or truncated uORF by mutation of the ATG start to CTG again lead to downstream ER-GFP expression greater than the basic ER-GFP construct (track 4 vs tracks 1&3), *i.e.* there was release from, translational repression. Note that the transfections shown in Fig. 4 used 1μg of the test construct and 1μg of the HEM45-GFP reference, we have since doubled the level of constructs and eliminated carrier DNA to achieve better reference expression. The overall extent of inhibitory effects measured in western blots tend to be larger than in the flow cytometry assay. We are looking at the total cell population in immunoblots whereas we gate out cells with basal auto-fluorescence (seen with *all* cells) in the flow data analysis and are aware that fraction of cells contributing to the measured signal in flow cytomety falls with transfectants of the weaker GFP-expressing constructs. We achieve a fair degree of agreement from the two assays if we factor in the fraction of cells gated in the flow cytometry assay.

These data clearly indicate that the proximal uORF and modified uORF are effective in inhibiting downstream ER-GFP expression in multiple cells backgrounds in addition to providing an alternate assay to confirm that the uORFs are regulatory.

Task 2. *Vary the spacing between the main ER translational start and the terminator of the prox uORF. Decrease and increase the spacing by looping in/out regions using modified PCR primers. Carry out studies using either GFP fusions or use the entire ER as reporter.*

A series of constructs varying spacing between the uORF and ER main translational starts have been made in the context of our ER-GFP construct. 15, 30 and 40 nt sequences were looped out in the 52 nt inter-cistronic space between the uORF and the main ER translational start, adjacent to the uORF terminator (see Fig. 6 cartoon). The effect on ER-GFP expression was assessed by western blot analysis. Results were equivocal (see blot data in Fig 6) as GFP expression from the construct with the largest deletion was *increased* compared to the parental ER-GFP construct while the expression from the others was l*ower*. For western blot analysis we need to repeat the transfections with the HEM45-GFP transfection reference construct to establish equivalent transfection has occurred. Again it may be experimentally easier to use the truncated form of the uORF which is more inhibitory as a starting point. A point to make from current data is that spacing between

the uORF and main ER translational start does not appear crucial. Interpretation of these studies will always potentially difficult as mechanism could be based on the separation of the two translational starts or could be related to the need for translational re-initiation prior to translation of the down stream ER-GFP. These two events are related, but mechanisms could exist that are more related to one aspect rather than the other.

Task 3. *Generate expression constructs for full length ER protein containing 5' regions of both the proximal and distal constructs, introduce to ER negative cells and assess relative efficiency of ER expression using western analysis, determine extent of regulatory effects on ER levels in comparison to, say, estradiol treated cells (estrogen tends to destabilize its receptor). Include, as controls, similar constructs where the uORF translational starts have been eliminated (ATG to AAG mutation). Extend studies to ER regulated reporter constructs i.e. use the modified ER expression constructs to modulate expression of luciferase constructs carrying estrogen response elements. Finally, time permitting, determine if the regulatory effects of the proximal uORF are similar in both normal immortalized lines (eg MCF-10) and breast tumor lines (eg MDA-MB-231).*

The Prox-ER constructs are now available for these studies we need to make the equivalent distal ER constructs. A cDNA expression construct in pRC-CMV was modified to make the transcript specific ER - expression constructs. Prox transcripts extending from upstream regions 5' to the uORF to past a Not 1 sites internal to the main ER ORF in the transcript were prepared . The fragments were inserted into the ER construct using the internal Not 1 site and an upstream restriction site included in forward PCR primers and present in the cloning vector polylinker. A second version of this construct is available were the uORF start has been eliminated (the forward PCR primer modified the ORF ATG to CTG). The distal constructs will also be in the coming year and we may modify the prox uORF to make the more potent C-terminal (Phe to stop) truncated ORF peptide.

Note that the Western immunoblot studies of ER-GFP constructs in various cell lines (see task 1 narrative) suggest that the uORF action is similar in all cells. The salient difference between tumors will probably be the relative use of the alternate ER promoters, and the differing translational potential of the alternate transcripts.

We have requested a no-cost extension for a second year as the work has been done by Mingfei Luo, a student, who also had major course requirements to fulfill and this slowed progress.

Key accomplishments

* We have established that C-terminal codons of the uORF mask the full potential of the uORF of the ER proximal promoter transcript to inhibit downstream translation of ER as estimated from an ER-GFP fusion reporter.

* We have delineated a small region of the uORF that should be the core of the sequence motif that primarily mediates translational inhibition of downstream expression of ER by the ER prox-uORF

* We havedeveloped an approach for normalizing expression in Western immunoblots of GFP transfectants using a GFP fusion construct created for a prior project.

* We demonstrated that the ER prox-uORF and some of its derivatives are functional in multiple cell environments.

Reportable Outcomes

The preliminary data from this project were included in an abstract to the fall 2002 Era of Hope meeting, per contract requirements. Ms Luo has also prepared, based on the data in this report, a Pre-doctoral fellowship application (BC021821) for the (June) fiscal 2002 competition of the Breast Cancer Research program..

Conclusions

We have made several clear observations about control of downstream ER expression by the prox ER transcript uORF. We have shown that C-terminal residues masks the full inhibitory potential of the uORF and a region of several codons has been defined as the most likely source of inhibitory effects.

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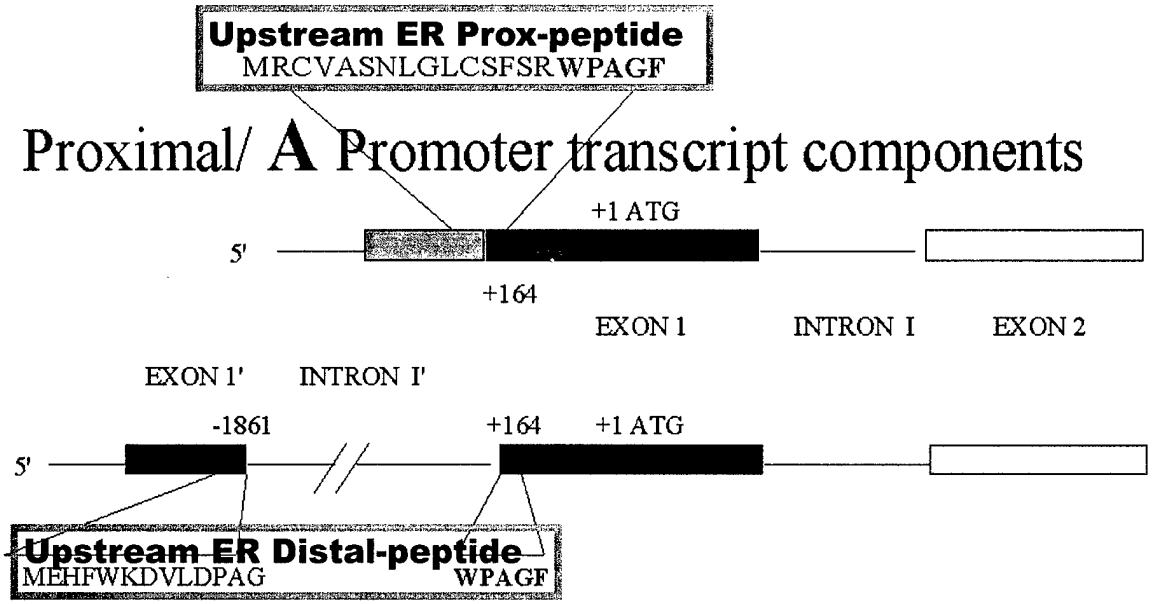


Fig. 1. Organization of the 5' regions of the human ER-alpha gene: Two promoters (distal [B, lower] and proximal [A, upper]) are present in the ER- gene and utilized in reproductive tissues. Alternative promoter transcripts converge at nt 164 of the mature mRNA (numbering based on proximal promoter transcript) and have a common ER coding region. Short ORF's are present in the upstream 5' region, these differ in the their N-terminal regions but share 5 C-terminal residues, by (RNA) splicing. Peptide sequences are shown

Region	DNA Sequence and Potential Translational Product
Distal 5' uORF	MetGluHisPheTrpLysAspValLeuAspProAlaGly**TrpProAlaGlyPheTer** ..caagcccATGGAACATTTCTGGAAAGACGTTCTTGATCCAGCAGG**GTGGCCCGCCGGTTTCTGAgcc...**
Proximal 5' uORF	1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 MetArgCysValAlaSerAsnLeuGlyLeuCysSerPheSerArg**TrpProAlaGlyPheTer** ..gcgggacATGCGCTGCGTCGCCTCTAACCTCGGGCTGTGCTCTTTTTCCAG**GTGGCCCGCCGGTTTCTGAgcc.**
Major ER ORF	MetThrMetThrLeuHisThrLysAlaSerGlyMetAlaLeuLeuHisGlnIle .... ccacggaccATGACCATGACCCTCCACACCAAAGCATCTGGGATGGCCCTACTGCATCAGATCCA...
Kozak Optimal Translational Start	Met ... gccgccRccATGG...

Fig. 1B Translational Start Regions of Estrogen Receptor Open Reading Frames
The DNA sequences and entire potential encoded peptides of alternate ER transcript upstream regions are shown together with the fragment of the main ER coding region that is included in ER-GFP fusion constructs. The DNA sequences 5' of the translational starts are shown and are included in constructs as they contribute to the relative strength of translational start sites. The five shared codons (from a common exon) of the proximal and distal transcripts are shown in bold. For comparison the optimal translational start defined by Kozak (REF) is included; the critical elements of the ATG flanking sequences are the purine (R) at -3 and the guanine residue at +4.

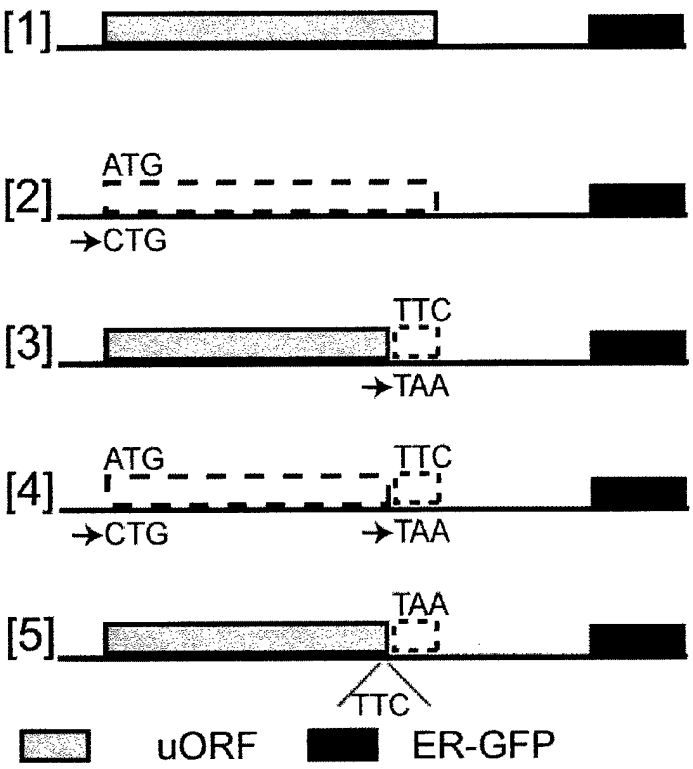


Fig.2: Cartoon of ER-GFP constructs

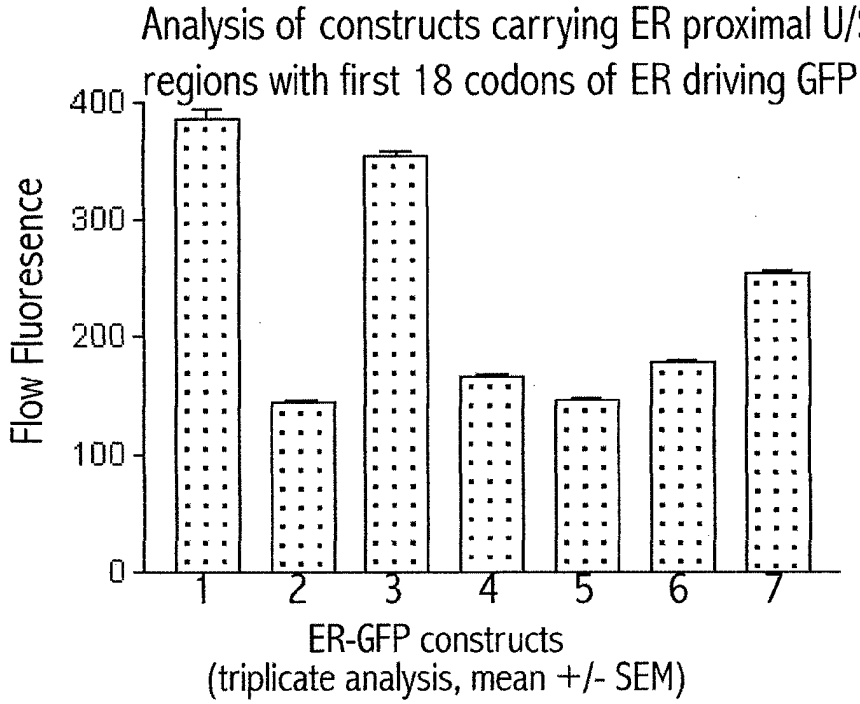
Construct **[1]**: containing wild type proximal ER upstream regions and main ER ORF sequence (18 AA)-GFP fusion.

Construct **[2]**: same as **[1]** except the uORF translational start ATG is eliminated by mutation to CTG

Construct **[3]**: same as **[1]** except uORF C-terminal Phe mutated to a termination codon (TTC->TAA)

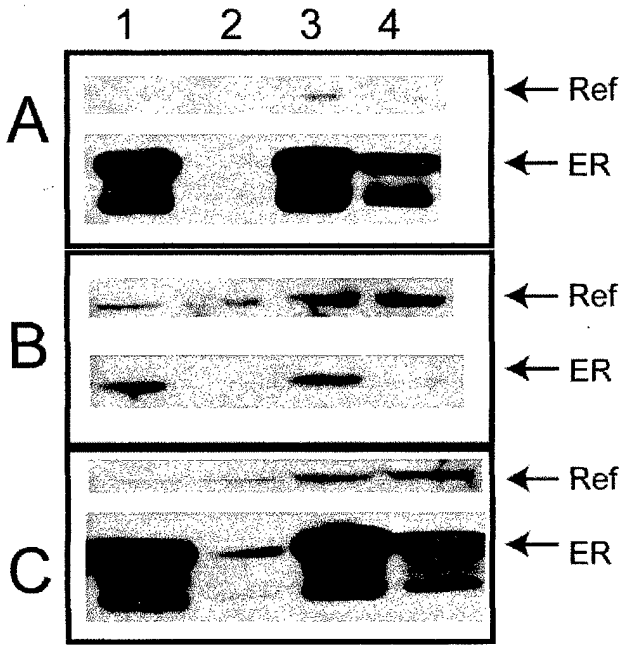
Construct **[4]**: same as **[3]** except the uORF translational start ATG is eliminated by mutation to CTG

Contruct **[5]**: same as **[1]** except uORF C-terminal Phe is looped out



1. Proximal ER-GFP construct as 7, but U/S ATG->CTG (construct **[2]** in Fig 2)
2. Proximal ER-GFP construct as 7, but U/S ORF last codon was mutated from Phe (TTC) to Stop (TAA) (this is construct **[3]** in Fig 2.)
3. Proximal ER-GFP construct as 7, but U/S ORF ATG->CTG, and last Phe ->STOP (**[4]** in Fig .2)
4. Proximal ER-GFP construct as 7, but U/S ORF ATG->CTG, and introduced ATG at codon 15
5. Proximal ER-GFP construct as 7, but U/S ORF ATG->CTG. and introducted ATG at codon 10
6. Proximal ER-GFP construck as 7, but U/S ORF codon 19 was mutated from Gly (GGT) to Stop (TGA)
7. Proximal ER-GFP construct (ER proximal transcript, 7 bp 5' of the U/S ORF ATG to 56 bp 3' of first 18 ER codons , fused in frame to GFP) This is construct **[1]** in Fig 2.

Fig. 3. ER proximal uORF transcript sequences modify expression of an ER-GFP reporter cassette



A: ISHIKAWA B: MCF-7 C: MDA-MB 231

1. Proximal ER-GFP mt (U/S ATG->CTG, **[2]** in Fig.2)
2. Proximal ER-GFP mt (U/S last Phe->Stop, **[3]** in Fig.2)
3. Proximal ER-GFP mt (U/S ATG-> CTG and last Phe -> Stop, construct **[4]** in Fig. 2)
4. Proximal ER-GFP (construct **[1]** in Fig. 2)

Fig. 4. Western blot analysis of ER-GFP expression in transiently transfected Ishikawa MCF-7 and MDA-MB 231 cells using anti-GFP Ab. Reference signal is generated from a contransfected invariant HEM45-GFP construct.

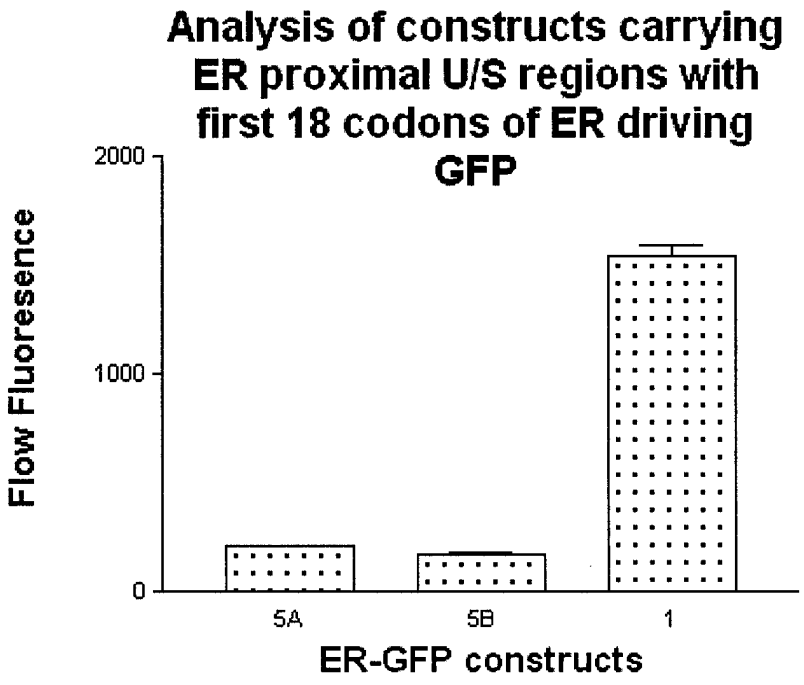


Fig 5: Flow Cytometry Analysis of GFP expression from constructs carrying our basic prox ER-GFP fusion (item 1 here and in fig 2 cartoon) and constructs where the c-terminal Phe residue of the upstream uORF has been eliminated by looping out (two independent clones analyzed, 5A and 5B, also see item 5 cartoon fig 2). The Phe residue here was eliminated by removing a complete codon. the data suggests that c-terminal residues modulate inhibitory action of the ORF. the effect in the to Phe to stop mutant in earlier figures was not primarily due to introduction of an extra terminator.

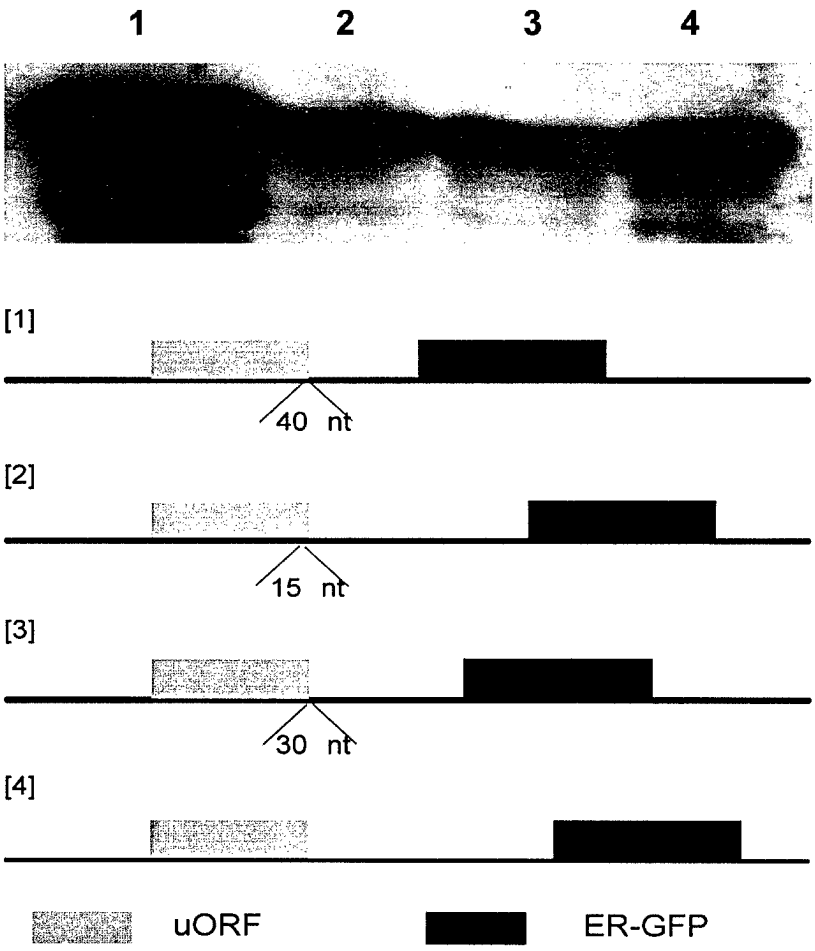


Fig. 6: Top panel shows western blot analysis of ER-GFP expression in transiently transfected cells. Rabbit polyclonal GFP antibody is used and the protein is resolved by 12% polyacrylamide gel. Lower panel is the cartoon of GFP constructs
Construct [1]: same as [4] except the space between proximal ER upstream region and main ER ORF is decreased by 40 nts
Construct [2]: same as [4] except the space between proximal ER upstream region and main ER ORF is decreased by 15 nts
Construct [3]: same as [4] except the space between proximal ER upstream region and main ER ORF is decreased by 30 nts
Construct [4]: contain wild type proximal ER upstream regions and main ER ORF sequence (18 AA)-GFP fusion. The uORF terminates 52 nt 5' to the main ER start